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(71) Applicant (for all designated States except US): VIRCO N.V. [BE/BE]; Drie Eikenstraat 661, B-2650 Edegem (BE).

(72) Inventors; and
(75) Inventors/Applicants (for US only): DE BETHUNE, Marie-Pierre [BE/BE]; Tweeleeuwenstraat 15, B-3078 Everberg (BE). HERTOGS, Kurt [BE/BE]; Sint Vincentiusstraat 53, B-2018 Antwerpen (BE). PAUWELS, Rudi [BE/BE]; Damstraat 166, B-1982 Weerde (BE).

(74) Agent: RYAN, Anne; Anne Ryan & Co., 60 Northumberland Road, Ballsbridge, Dublin 4 (IE).

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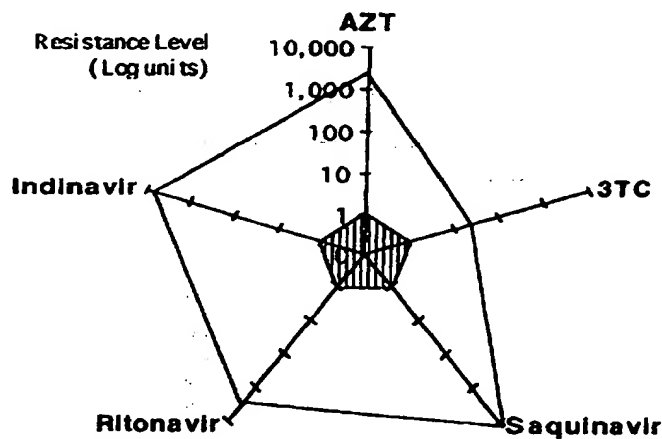
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(54) Title: METHOD OF MANAGING THE CHEMOTHERAPY OF PATIENTS WHO ARE HIV POSITIVE BASED ON THE PHENOTYPIC DRUG SENSITIVITY OF HUMAN HIV STRAINS

(57) Abstract

A method of managing HIV chemotherapy of patients who are HIV positive comprises transfecting a cell line susceptible to infection by HIV with a sequence, preferably that coding for RT and protease, from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which the sequence has been deleted, culturing the transfected cells so as to create a stock of chimeric viruses, assessing the phenotypic sensitivity of the chimeric viruses to an inhibitor of the enzyme encoded by the pol gene of HIV and assigning a value thereto, constructing a data set comprising the value for chimeric virus sensitivity and the corresponding value for a chimeric wild-type strain of HIV, repeating the sensitivity assessment for at least two further inhibitors and thereby constructing at least three such data sets in total, representing the data sets in two-dimensional or three-dimensional graphical form such that the difference between the chimeric and wild-type sensitivities in the case of each data set provides a visual measure of the resistance of the chimeric stock to treatment by the inhibitor in question, and selecting the optimum inhibitor(s) on the basis of the graphical representation of the resistances so measured. The method yields phenotypic information on individual HIV infected patients on a large scale, economically and rapidly. The method is applicable to all currently available chemotherapeutic regimens and it is expected to be equally applicable to future chemotherapeutic regimens.

Resistance Level (Log units)

Maximal Measurable Resistance
Patient Sample Actual Resistance
Reference Strain IIIB

Description

Method of managing the chemotherapy of patients who are HIV positive based on the phenotypic drug sensitivity of human HIV strains

Technical Field

5   The present invention relates to a method of managing the chemotherapy of patients who are HIV positive, as well as a clinical management device for use by physicians treating such patients based on the phenotypic drug sensitivity of human HIV strains for inhibitors of one or more enzymes of the pol gene of HIV, as well as a method for

10   simultaneously determining the phenotypic drug sensitivity of two or more of the enzymes of the pol gene of HIV to inhibitors thereof.

Background Art

         To date, several chemotherapeutic regimens have been developed for treating HIV infected patients. Certain of these regimens have been

15   approved for clinical use, and others are the subject of on-going clinical trials. It can be assumed that the number of approved chemotherapeutic regimens will increase steadily in the near future. Increasingly, combination therapy or multiple drug treatment regimens are being used because of the development of drug-resistant HIV variants during

20   therapy. Although these chemotherapeutic regimens have been shown to exert an effect on virological (viral load), immunological and clinical parameters of HIV disease, practical experience teaches that these effects are transient. In particular, one finds that the HIV strains infecting an individual patient after a while start to display reduced sensitivity to the

25   drug or drug combination with which said patient is being treated. The loss of efficacy of the chemotherapy can vary from patient to patient, from drug to drug, or from drug combination to drug combination. It is well established that the loss of efficacy to a particular type of chemotherapy can be associated with a genotypic pattern of amino acid

30   changes in the genome of the HIV strains infecting the patient. This probably renders these HIV strains less susceptible to the chemotherapy.

As an HIV infected patient is exposed to several chemotherapeutic regimens over extended periods of time, more complex patterns of amino acid changes in the genome of infecting HIV strains occur which for the present defeat a rational approach to the further treatment of the

5   infected patient. As implied in the previous explanation, one can routinely determine the genotypic changes occurring in HIV strains exposed to different chemotherapeutic regimens involving single or multiple anti-HIV drugs, but thus far it has proven very difficult to derive from these data information enabling a physician in charge of

10   prescribing the chemotherapy whether or not it is sensible to initiate or continue a particular chemotherapeutic regimen. In other words, the genotypic information which is available on a limited scale, cannot routinely be translated into phenotypic information enabling the responsible physician to make the crucial decision as to which

15   chemotherapy a patient should preferably follow. The problem also exists for drug-naive patients who become infected by drug-resistant HIV strains.

       Viral load monitoring is becoming a routine aspect of HIV care. However, viral load number alone cannot be used as a basis for deciding

20   which drugs to use alone or in combination.

       Combination therapy is becoming increasingly the chemotherapeutic regimen of choice. When a person using a combination of drugs begins to experience drug failure, it is impossible to know with certainty which of the drugs in the combination is no

25   longer active. One cannot simply replace all of the drugs, because of the limited number of drugs currently available. Furthermore, if one replaces an entire chemotherapeutic regimen, one may discard one or more drugs which are active for that particular patient. Furthermore, it is possible for viruses which display resistance to a particular inhibitor to

30   also display varying degrees of cross-resistance to other inhibitors.

       Ideally, therefore, every time a person has a viral load test and a viral load increase is detected, a drug sensitivity/resistance test should

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also be carried out. Until effective curative therapy is developed, management of HIV disease will require such testing.

Currently there does exist a phenotyping method which is based on virus isolation from plasma in the presence of donor peripheral blood

5   mononuclear cells (PBMCs), and subsequent phenotyping in said cells (Japour, A.J., *et al.* (1993) Antimicrobial Agents and Chemotherapy; Vol. *37*, No. 5, p1095-1101). This co-cultivation method, which is advocated by the AIDS Clinical Trial Group (ACTG) - particularly for phenotyping AZT (synonymous herein with zidovudine/Retrovir

10   (Retrovir is a Trade Mark)) resistance, is time-consuming, costly and too complex to be used on a routine basis.

A phenotypic recombinant virus assay for assessment of drug susceptibility of HIV Type 1 isolates to reverse transcriptase (RT) inhibitors has been developed by Kellam, P. and Larder, B.A.

15   (Antimicrobial Agents and Chemotherapy (1994) Vol. *38*, No. 1, p23-30). This procedure allows the generation of viable virus by homologous recombination of a PCR-derived pool of RT coding sequences into an RT-deleted, noninfectious proviral clone, pHIVΔRTBstEII. Analysis of two patients during the course of

20   zidovudine therapy showed that this approach produced viruses which accurately exhibited the same genotype and phenotype as that of the original infected PBL DNA. However, the procedure involves isolation of the patient virus by co-cultivation of patient plasma or patient PBMCs with donor PBMCs. Such prior cultivation of virus may distort the

25   original virus composition. Furthermore, this method, although allowing one to determine the sensitivity of the isolates to various inhibitors, does not provide the physician with information as to whether to continue with the existing chemotherapeutic regimen or to alter the therapy.

Also when one enzyme only of the pol gene is being studied, the

30   method does not readily lend itself to routine phenotypic assessment of combination therapy which conventionally involves the use of one protease and 2 RT inhibitors.

The nested PCR (polymerase chain reaction) procedure used in the recombinant virus assay can lead to a situation where the recombinant virus does not truly reflect the situation with the HIV strains infecting the patient under investigation. This problem resides in DNA sequence

5   homology and the minimum amount of homology required for homologous recombination in mammalian cells (C. Rubnitz, J. and Subramini, S. (1984) Molecular and Cellular Biology Vol. 4, No. 11, p2253-2258). Accordingly, any phenotypic assay based on the recombinant virus approach should endeavour to ensure that as much as

10   possible of the patient material is amplified and that there is maximum recombination.

Thus, the RNA extraction and nested PCR procedures employed should ensure that the viral genetic material is amplified such that the amplified material maximally reflects the viral genetic diversity in the

15   patient being investigated.

In current clinical practice there is therefore a hard-felt need (a) to determine rapidly and on a routine basis the phenotypic drug sensitivity of HIV strains infecting a particular patient, (b) to process the thus obtained data into easily understood information, and (c) to initiate,

20   continue or adjust on the basis of said information the chemotherapy prescribed for said particular patients.

Disclosure of the Invention

According to a first aspect of the invention there is provided a method of managing HIV chemotherapy of patients who are HIV

25   positive, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses, assessing the phenotypic sensitivity of said chimeric

30   viruses to an inhibitor of said enzyme encoded by the pol gene of HIV and assigning a value thereto, constructing a data set comprising said value for chimeric virus sensitivity and the corresponding value for a

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chimeric wild-type strain of HIV, repeating the sensitivity assessment for
at least two further inhibitors and thereby constructing at least three such
data sets in total, representing said data sets in two dimensional or three
dimensional graphical form such that the difference between the
5   chimeric and wild-type sensitivities in the case of each data set provides
a visual measure of the resistance of the chimeric stock to treatment by
the inhibitor in question, and selecting the optimum inhibitor(s) on the
basis of the graphical representation of the resistances so measured.

The method according to the invention yields phenotypic
10   information on individual HIV infected patients on a large scale,
economically and rapidly. The method is applicable to all currently
available chemotherapeutic regimens and it is expected to be equally
applicable to future chemotherapeutic regimens.

The method according to the invention provides the physician
15   with phenotypic data on patient HIV strains which can be immediately
used to determine whether a particular chemotherapeutic regimen should
be initiated, continued or adjusted.

Preferably, the data sets are represented on a polygonal or quasi-
circular graph comprising:

20   (a)   a plurality of normalised axes extending radially from an
origin, each axis corresponding to one data set or inhibitor
or combination thereof;

(b)   the axes being normalised such that the sensitivity values
for wild-type HIV for the various inhibitors are equal on
25   each axis, the data points for wild-type HIV being
optionally represented and connected to form a regular
polygon whose vertices lie on the axes and whose center is
defined by the origin;

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(c)   on each axis a data point representing the sensitivity value of the chimeric HIV stock against the inhibitor corresponding to said axis is plotted, the chimeric data points being optionally connected to form a regular or irregular polygon the shape of which represents the resistance of the chimeric stock to a range of inhibitors.

A polygonal or quasi-circular graph provides the advantage that the patient's resistance to a number of drugs is characterised in terms of the degree of divergence between the polygon representing the patient's chimeric HIV stock and the polygon representing the wild-type strain. The areas of the polygons will generally diverge more in some areas than in others, indicating in each case a greater or lesser degree of resistance to the inhibitor whose axis passes through the area in question.

Thus, the method according to the invention takes a chimeric HIV stock and provides a map of the resistance of this stock across a range of inhibitors. In this way the map or graph provides a technical characterisation of an aspect of the chimeric stock which is not obtained by conventional measurements.

In a preferred embodiment, the normalised axes are equiangular from one another.

Further, preferably, each axis has a logarithmic scale.

Further, preferably, eccentric data points in the chimeric polygon, if represented, identify inhibitors whose usefulness can be assumed to be of little or no benefit to the patient, while data points lying within, on or close outside the wild-type polygon identify inhibitors whose usefulness can be assumed to be of substantial benefit to the patient.

When worst case values are represented along with the chimeric and wild-type HIV, a usually irregular polygon encloses the chimeric and wild-type polygons. The meaning of the term "eccentric" as used above denotes a data point lying relatively close to the worst-case border

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and relatively far from the wild-type polygon. Similarly the term "close outside the wild-type polygon" refers to relative closeness to the wild-type polygon when compared to the distance from the worst-case border.

5   The method as hereinabove defined is limited in the sense that the measurable resistance against an inhibitor is dependent on the particular range of concentrations of the inhibitor used. Also one must endeavour to reduce the effects of biological variability. Accordingly, it is desirable to obtain a value for maximum or worst-case measurable resistance where it is assumed that a given inhibitor has no effect. This

10   concentration, e.g. 100 μM, is generally the maximum concentration that can practically be tested, but may also be derived from e.g. pharmacological, toxicological or pharmacokinetic studies. The comparison of the resistance level of the patient under investigation and the maximum measurable resistance determines what is the significant

15   level of resistance for the patient under investigation. The maximum measurable resistance and the actual resistance can be suitably shown on a bar graph as hereinafter described.

In a still further preferred embodiment of the invention each of said three or more data sets further comprises a value for worst-case

20   measurable resistance for the inhibitor in question, said worst case values being represented on said graphical representations such that the data point for the chimeric stock can be compared both to wild-type and to worst-case HIV, thereby providing an assessment of the relative value of the inhibitor in a particular case.

25   Experiments with in excess of 150 patient samples have revealed a close correlation between resistance development and therapy history as hereinafter further illustrated in the Examples. A close correlation has been found with the data generated in accordance with the invention relative to classical virus isolation and phenotyping techniques.

30   The method in accordance with the invention can thus be used for an individualised and more rational management of HIV chemotherapy. Thus, use of the method according to the invention in combination with

the proper administration of anti-HIV drugs should ultimately lead to better treatment and survival of patients infected with the HIV virus.

The method according to the invention has particular application where an individual patient has been receiving many different drugs and his mutation pattern is not readily interpreted by attending physicians.

According to a further aspect of the invention there is provided a method of managing HIV chemotherapy of patients who are HIV positive, which comprises the steps of:

(a)   periodically assessing the phenotypic sensitivity of a patient's HIV strains by a method hereinabove described;

(b)   maintaining the chemotherapy with the selected inhibitor while the patient's HIV strains remain susceptible to the selected chemotherapy;

(c)   selecting a different inhibitor if and when the susceptibility of the original inhibitor decreases.

According to a still further aspect of the invention there is provided a clinical management device for use in the management of chemotherapy of patients who are HIV positive, said device bearing a graphical representation of a plurality of data sets as hereinabove defined.

We have coined the term "Antivirogram" for the clinical management device according to the invention and this term will be used hereinafter in the specification. This device provides the physician with a clear representation of the relative changes and susceptibilities for different inhibitors which are or which may be used in the clinical management of individual HIV-infected patients.

By HIV herein is generally meant HIV-1. However, the invention is also applicable to HIV-2.

Preferably, the phenotypic sensitivity of said chimeric viruses to inhibitors of at least two enzymes encoded by the pol gene of HIV is simultaneously assessed.

In a further aspect of the invention there is provided a method of determining the phenotypic drug sensitivity of individual HIV strains in a patient to inhibitors of at least two enzymes encoded by the pol gene of HIV, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses and assessing the phenotypic sensitivity of said chimeric viruses to inhibitors of said enzymes encoded by the pol gene of HIV.

The desired sequence from the pol gene is isolated from a sample of a biological material obtained from the patient whose phenotypic drug sensitivity is being determined. A wide variety of biological materials can be used for the isolation of the desired sequence.

Thus, the biological material can be selected from plasma, serum or a cell-free body fluid selected from semen and vaginal fluid. Plasma is particularly preferred and is particularly advantageous relative to the use of PBMCs as used in the prior art described above.

Alternatively, the biological material can be whole blood to which an RNA stabiliser has been added.

In a still further embodiment, the biological material can be a solid tissue material selected from brain tissue or lymph nodal tissue, or other tissue obtained by biopsy.

As hereinafter demonstrated, when a biological material such as plasma is used in the isolation of the desired sequence, a minimal

volume of plasma can be used, typically about 100-250µl, more particularly of the order of 200µl.

Further, preferably the two enzymes selected will be selected from HIV RT, protease and integrase.

5   Viral RNA is conveniently isolated in accordance with the invention by methods known *per se*, for example the method of Boom, R. *et al.* (Journal of Clinical Microbiology (1990) Vol. *28*, No. 3, p.495-503).

In the case of plasma, serum and cell-free body fluids, one can

10   also use the QIAamp viral RNA kit marketed by the Qiagen group of companies.

Preferably, the cell line susceptible to infection by HIV is a CD4$^+$ T-cell line.

Further, preferably, the CD4$^+$ T-cell line is the MT4 cell line or

15   the HeLa CD4$^+$ cell line.

Reverse transcription can be carried out with a commercial kit such as the GeneAmp Reverse Transcriptase Kit marketed by Perkin Elmer.

The desired region of the patient pol gene is preferably reverse

20   transcribed using a specific downstream primer.

In the case where the sequence to be reverse transcribed is that coding for reverse transcriptase or reverse transcriptase and protease, the downstream primer is preferably OUT3 : 5'-CAT TGC TCT CCA ATT ACT GTG ATA TTT CTC ATG-3' (SEQ ID NO: 1).

25   In a particularly preferred embodiment a patient's HIV RT gene and HIV protease gene are reverse transcribed using the HIV-1 specific OUT 3 primer and a genetically engineered reverse transcriptase lacking

11

RNase H activity, such that the total RNA to be transcribed is converted
to cDNA without being degraded. Such a genetically engineered reverse
transcriptase, the Expand (Expand is a Trade Mark) reverse
transcriptase, can be obtained from Boehringer Mannheim GmbH.

5   Expand reverse transcriptase is a RNA directed DNA polymerase.
The enzyme is a genetically engineered version of the Moloney Murine
Leukaemia Virus reverse transcriptase (M-MuLV-RT). Point mutation
within the RNase H sequence reduces the RNase H activity to below the
detectable level. Using this genetically engineered reverse transcriptase
10   enables one to obtain higher amounts of full length cDNA transcripts.

Following reverse transcription the transcribed DNA is amplified
using the technique of PCR.

Preferably, the product of reverse transcription is amplified using
a nested PCR technique.

15   Preferably, in the case where the region of interest is the RT
region, a nested PCR technique is used using inner and outer primers as
described by Kellam, P. and Larder, B.A. (1994 *supra*). When the
region of interest is that spanning the RT and protease genes, the specific
primers used are preferably a combination of OUT 3/IN 3 (downstream)
20   and RVP 5 (upstream).

The primer RVP 5 (Maschera, B., *et al.* Journal of Virology, *69*,
5431-5436) has the sequence 5'-GGGAAGATCTGGCC
TTCCTACAAGGG-3' (SEQ ID NO: 2).

A schematic representation of the amplification is set forth in Fig.
25   3 and is described in greater detail in Example 2.

The amplification of the protease cDNA actually involves a hemi-
nested PCR procedure as will be apparent from Fig. 3.

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The nested PCR technique has the advantage over the known simple PCR techniques in that it enables one to obtain the most specific PCR product.

However, to obtain an even higher fidelity and yield during PCR,
5   one can make use of a mixture of thermostable polymerases (Barnes, W.M. (1994) Proc. Natl. Acad. Sci. U.S.A. *91*, 2216-2220). Such a polymerase mixture is available from Boehringer Mannheim GmbH, namely the Expand (Expand is a Trade Mark) high fidelity PCR system. Using this system we have obtained increased sensitivity, namely a
10   sensitivity which is ten times or greater than that obtained with a conventional PCR procedure using Taq polymerase alone.

When the region of the pol gene is that embracing the RT and protease genes, preferably the HIV-DNA construct is one from which the RT and protease genes are deleted and is the plasmid pGEMT3-
15   ΔPRT as deposited at the Belgian Coordinated Collections of Microorganisms-BCCM LMBP-Collection on November 8, 1996 under the number LMBP3590.

However, several approaches can be adopted to generate a plasmid containing the HIV-1 provirus carrying a deletion for the
20   protease as well as for the RT gene. One possibility is the introduction of the desired deletion by means of oligonucleotide-mediated mutagenesis. However, the procedure adopted hereinafter in Example 2 involves the generation of the desired construct by making use of specific restriction enzymes and subcloning procedures, as hereinafter
25   described. Although the final results depend on the available restriction sites a major advantage of this procedure is that one can obtain conclusive results rapidly.

To ensure the most efficient outcome for the transfection, the PCR-product, being transfected, should ideally be purified by anion
30   exchange spin columns in a manner known *per se.* A suitable kit is the QIAquick PCR Purification Kit marketed by the Qiagen group of companies.

13

Transfection can be achieved by electroporation or, alternatively, by the use of lipids, especially cathionic lipids, DEAE dextran, $CaHPO_4$, etc.

5   In the case of lipid transfection one can avail of a PERFECT (PERFECT is a Trade Mark) transfection kit marketed by Invitrogen B.V. of Leek, the Netherlands.

Thus, for transfection an HIV-DNA construct from which the gene or genes of choice from the pol gene has/have been deleted is used in conjunction with the product obtained following amplification.

10   The construct can be the plasmid pHIVΔRT (obtainable from the Medical Research Council (MRC)) if it is the RT gene only that is deleted. When the RT and protease genes are both deleted a suitable HIV DNA construct is the plasmid pGEMT3-ΔPRT described herein and which is a high copy vector. Such plasmids are linearised prior to 15   transfection according to methods known *per se*.

A particular advantage of using a construct coding for more than one pol gene enzyme, for example a ΔPRT construct, is that one is more likely to include more of the original patient material in the construct than if a single gene is used, so that the amplified material reflects to a 20   greater extent the viral genetic diversity in the particular patient being investigated.

It will be appreciated that it is preferable that the specific primers selected for the nested PCR are located outside the body sequences of the target enzymes to be amplified and investigated. It will furthermore 25   be appreciated that a combination of RT and protease is likely to provide better results for studying RT than RT alone, because forty more amino acids are patient borne relative to the situation with RT alone. For studying the protease, one should be aware that the first nine amino acids of the protease are still derived from the construct's (pGEMT3-ΔPRT) 30   wild-type backbone.

14

When the transfection of the cells is achieved through electroporation, the parameters selected are optimized to achieve good cell growth and virus production. The electroporation can conveniently be conducted at approximately 250μF and 300V. Preferably, the

5   electroporation is conducted in the presence of about 10μg of linearised plasmid e.g. pHIVΔRTBstEII and about 5μg of amplified PCR product e.g. RT PCR product. Upon successful intracellular homologous recombination, new chimeric HIV is formed within 5 to 10 days. With known techniques typical cultivation times are 12-14 days before

10   chimeric HIV is formed. Culture supernatant aliquots are stored at - 70°C or lower temperatures.

It is readily seen that one can use the above methods for isolating and amplifying other HIV genes, e.g. the integrase gene, or more than one other HIV gene, e.g. both the RT and the integrase gene, and

15   transfecting a CD4$^+$ T-cell with the respective integrase or RT/integrase PCR products in conjunction with an appropriate linearised HIV-DNA construct from which the relevant gene (or genes) is deleted.

The newly formed chimeric viruses are titrated and then analysed for their phenotypic sensitivity (i.e. susceptibility) to the different pol

20   gene enzyme inhibitors, preferably in an automated cellular-based assay.

Preferably, the phenotypic drug sensitivity of the chimeric viruses and of the wild HIV strain, which is suitably a recombinant wild HIV strain, to one or more RT, protease or integrase inhibitor(s) is expressed as an inhibitory concentration (IC value).

25   The susceptibilites of the chimeric viruses and of the wild type HIV strain to one or more RT inhibitors and/or one or more protease inhibitors and/or one or more integrase inhibitors can be expressed as for example 50% or 90% inhibitory concentrations (IC$_{50}$ or IC$_{90}$ values).

Preferably, RT inhibitors are selected from nucleoside RT

30   inhibitors such as AZT, ddI (didanosine/Videx (Videx is a Trade Mark), ddC (zalcitabine), 3TC (lamivudine), d4T (stavudine), non-nucleoside

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RT inhibitors such as delavirdine (U 9051125 (BMAP)/Rescriptor
(Rescriptor is a Trade Mark)), loviride (alpha-APA), nevirapine (B1-RG-
587/Viramune (Viramune is a Trade Mark) and tivirapine (8-Cl-
TIBO(R86183)), protease inhibitors such as saquinavir, indinavir and

5   ritonavir and integrase inhibitors such as caffeic acid phenylethyl ester
(CAPE).

Suitable RT and/or protease inhibitors and/or integrase inhibitors
are selected from nucleoside RT inhibitors such as AZT, ddI, ddC, 3TC,
d4T, 1592U89 and the like, non-nucleoside RT inhibitors such as

10   loviride, nevirapine, delaviridine, ateviridine, and tivirapine (8-Cl TIBO)
and the like, protease inhibitors such as saquinavir, indinavir and
ritonavir and the like, and integrase inhibitors such as caffeic acid
phenylethyl ester (CAPE) and HIV integrase inhibitors of the type
described in WO 95/08540 and GB 2,271,566.

15   The method according to the invention comprises the step of
comparing the phenotypic drug sensitivity of patient HIV strains with
one or more RT inhibitors and/or one or more protease inhibitors, and/or
one or more integrase inhibitors to that of a wild type HIV strain. For an
easy-to-understand representation of the relative changes in

20   susceptibility to the different drug compounds (or combinations) tested,
an Antivirogram graph, is constructed.

The graph should be interpreted as follows : eccentric data points
in the antivirogram identify chemotherapeutic regimens unlikely to
benefit the HIV infected patient any further, whereas data points within

25   or on the reference polygon, or only slightly beyond the reference
polygon, identify chemotherapeutic regimens likely to benefit the HIV
infected patient.

The methods according to the invention in combination with the
administration of the correct anti-HIV drugs should ultimately lead to

30   better treatment, improved quality of life and improved survival of HIV
infected patients ; i.e. ineffective treatment (due to the presence of or

16

emergence of resistant HIV strains) can be prevented or halted, and effective chemotherapy can be initiated in good time.

The present invention also concerns a clinical management device for use by physicians treating HIV infected patients comprising an
5   Antivirogram obtainable by the methods hereinbefore described.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the construction of the plasmid pGEMT3-ΔPRT;

Fig. 2 is a further and complementary schematic representation
10   of the construction of the plasmid pGEMT3-ΔPRT;

Fig. 3 is a schematic representation of that part of the HIV-HXB2D sequence containing protease and RT genes;

Fig. 4A-H is a complete sequence for that part of the HIV-HXB2D sequence containing protease and RT genes;

15   Fig. 5 is an Antivirogram for a patient harbouring 3TC resistant HIV strains as described in Example 5;

Fig. 6 is an Antivirogram for a drug-naive patient harbouring wild type like HIV strains as described in Example 6;

Fig. 7A is a bar graph showing relative change in drug
20   susceptibility for the patient of Example 7;

Fig. 7B is an Antivirogram for the patient the subject of Example 7;

Fig. 8A is a bar graph showing relative change in drug susceptibility for the patient of Example 8;

Fig. 8B is an Antivirogram for the patient the subject of
Example 8;

Fig. 9A is a bar graph showing relative change in drug
susceptibility for the patient of Example 9;

5   Fig. 9B is an Antivirogram for the patient the subject of
Example 9;

Fig. 10A is a bar graph showing relative change in drug
susceptibility for the patient of Example 10;

Fig. 10B is an Antivirogram for the patient the subject of
10   Example 10;

Fig.11A is a bar graph showing relative change in drug
susceptibility for the patient of Example 11;

Fig.11B is an Antivirogram for the patient the subject of
Example 11;

15   Fig.12A is a bar graph showing relative change in drug
susceptibility for the patient of Example 12; and

Fig.12B is an Antivirogram for the patient the subject of
Example 12.

The invention will be further illustrated by the following
20   Examples.

Modes for Carrying Out the Invention

Example 1

Protocol

1.   Extraction and amplification of viral RNA.

5   RNA was isolated from 100μl of plasma according to the method
described by Boom, R. *et al.* (1990, *supra*), and was reverse transcribed
with the GeneAmp reverse transcriptase kit (Perkin Elmer) as described
by the manufacturer and using an HIV-1 specific downstream primer
(OUT3 : 5'-CAT TGC TCT CCA ATT ACT GTG ATA TTT CTC ATC-
10   3'; SEQ ID NO: 1). PCR on reverse transcribed RNA was performed
with inner and outer primers as described by Kellam , P. and Larder, B.A.
(1994, *supra*). After chloroform extraction and centrifugation on
Centricon 100 columns or centrifugation on anion-exchange spin
columns (Quiagen), the isolated PCR product was ready for use in the
15   transfection reactions.

2.   Production and isolation of plasmid.

        Production of pHIVΔRT (MCR) plasmid was performed in *E. coli*.
Plasmid DNA was isolated from overnight cultures making use of Qiagen
columns as described by the manufacturer. Yield of the isolated plasmid
20   was determined spectrophotometrically by A260/280 measurement
(optical density measurement at $\lambda = 260$ and 280 nm). About 250 μg of
ultrapure plasmid DNA was obtained from 500 ml of bacterial culture.
The identity of the isolated plasmid was confirmed by restriction
analysis. Subsequently, the isolated plasmid DNA was linearised with
25   BstEII and purified again by a classical phenol/chloroform extraction.

3.   Transfection of cells.

        MT4 cells were subcultured at a density of 250,000 cells/ml before
transfection (exponential growth phase). Cells were pelleted and

resuspended in phosphate buffered saline (PBS) at a concentration of 3.1 10E6 cells/ml. A 0.8 ml portion (2.5 10E6 cells/ml) was used for each transfection. Transfection was performed with the Bio-Rad Gene pulser making use of 0.4 cm electrode cuvettes. Cells were electroporated in the

5   presence of 10μg of linearised pHIVΔRT plasmid and approximately 5μg of RT PCR product at 250μF and 300 V, followed by a 30-min incubation at room temperature. Subsequently, 10 ml of fresh culture medium was added to the cell suspension and incubation was performed at 37°C in a humidified atmosphere of 5% CO_2.

10   4.   Culture and follow-up of transfected cells.

During 7 to 10 days following the transfection, cells were monitored for the appearance of cytopathogenic effect (CPE). In the absence thereof, cells were subcultured in different flasks. Subsequently, culture supernatants of transfected cells were used to create a stock of

15   recombinant virus and stored in 1.5 ml aliquots at -70°C.

5.   Analysis of recombinant virus from patient viral RNA.

After titration of the new viruses, the stocks were used for antiviral experiments in the presence of serial dilutions of different HIV inhibitors. Titres of the harvested supernatants were determined by limited serial

20   dilution titration of virus in MT4 cells.

Viruses with a useful titre were used in antiviral experiments. For this purpose, 96-well microtitre plates were filled with 100μl of complete culture medium. Subsequently, stock solutions of compounds were added in 25μl volumes to series of duplicate wells. HIV- and mock-

25   infected cell samples were included for each drug (or drug combination). Exponentially growing MT4 cells were then transferred to the microtitre plates at a density of 150,000 cells/ml. The cell cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2. Five days after infection, the viability of the mock- and HIV-infected cells was

30   examined spectrophotometrically by the MTT method (Pauwels, R. *et al.* - J. Virol. Meth. (1988), *20* : 309-321) as described in Section 6 below.

20

6.   MTT assay.

To each well of the microtiter plates, 20μl of a solution of MTT
(7.5 mg/ml in PBS) was added. The plates were further incubated at
37°C for 1 h. Then, 150μl of medium was removed without disturbing
5   the MT4 cell clusters containing the formazan crystals. Solubilization of
the formazan crystals was achieved by adding 100μl 5% Triton X-100 in
acidified isopropanol (5 ml concentrated HCl per litre solvent).
Complete dissolution of the formazan crystals was obtained after the
plates had been placed on a plate shaker for 10 min. Finally the
10   absorbances were read at two wavelengths (540 and 690 nm). From
these optical density (OD) data, 50% inhibitory (IC_{50}) and 50% cytotoxic
(CC_{50}) concentrations were derived.

Example 2

Construction of a pHIVΔRTBstEII-variant with deletion of the HIV-1
15   protease and reverse transcriptase gene.

The protocol described in Example 1 was repeated, except that the
sequence of the HIV pol gene of interest was that coding for RT and
protease and the construct prepared was pGEMT3-ΔPRT as described
below. Other modifications relative to the procedure set out in Example
20   1 are set out below.

For amplification of viral RNA, reverse transcription from RNA to
DNA was again carried out with the OUT3 primer. However, for the
nested PCR procedure the primers used are as shown in Fig. 3. Thus, it
will be observed that the nested PCR procedure uses as outer primers
25   RVP5 and OUT3 and as inner primers RVP5 and IN3. Thus, this nested
procedure is, in effect a hemi-nested PCR procedure.

Production and isolation of pGEMT3-ΔPRT.

The final pGEMT3-ΔPRT construct is a derivative of pGEM9-
Zf(-) (Promega).

21

In short, the pGEMT3-ΔPRT construct is built up by introducing
the desired insert HIV-HXB2 (a protease and reverse transcriptase-
deleted proviral HIV-1 clone, including flanking human sequences) into
the XbaI restriction site of the vector pGEM9-Zf(-). The proviral genome
5   has been deleted from the AhdI site within the protease gene
(surrounding amino acid 9) to the BstEII site of the pHIVΔRTBstEII
construct (MRC Repository reference : ADB231). At the junction of the
ΔProRT deletion SmaI and BstEII sites are located which can be used for
linearisation of the proviral construct prior to transfection. The
10   construction of pGEMT3-ΔPRT is schematically represented in Figs 1
and 2. The yield of pGEMT3-ΔPRT was about 1mg out of 500ml
bacterial culture.

As indicated above, the plasmid pGEMT3-ΔPRT was deposited at
the Belgian Coordinated Collections of Microorganisms-BCCM LMBP-
15   Collection on November 8, 1996 under the number LMBP3590.

It was not expected that the introduction of the proviral genome
into another vector (pGEM9-Zf(-) instead of pIB120) would cause major
problems. pIB120, a derivative of pEMBL8(-) (according to information
provided by Kodak Scientific Imaging Systems), and pGEM9-ZF(-) are
20   similar vectors. Nevertheless the proviral vector pIB120HIV may be
unstable in recA+ E. coli host cells (Maschera, B., et al. J. Virol. (1995)
69, 5431-5436. Therefore the stability of the pGEMT3-ΔPRT construct
should be verified after every new preparation of plasmid.

HIV-HXB2 sequence:

25   The region of interest within the HIV-HXB2D sequence
(nucleotide 1800 to 4400) is represented in Fig. 3 (schematically) and
Fig. 4 (complete sequence). The location of several genes, restriction
sites, primers and deletions (ΔPro, ΔRT, ΔProRT) are also indicated.

The sequence of HIV-1 (isolate HXB2, reference genome, 9718bp)
30   was obtained from the National Center for Biotechnology Information

(NCBI), National Library of Medicine, National Institutes of Health *via* the ENTREZ Document Retrieval System.

Genbank name: HIVHXB2CG
Genbank Accesion No: Ø3455
NCBI Seq.ID No: 327742

Regions of recombination:

In combination with RT-PCR fragments generated by RVP5 and OUT3/IN3 primers, the pGEMT3-ΔPRT vector can be used to transfect MT4 cells as described in Example 1, Section 3. The region for recombination at the 5'-end of ΔProRT contains 188 nucleotides. The region for recombination at the 3'-end of ΔProRT is similar to the one described earlier (Kellam, P. and Larder, B.A. (1994) *supra*) and contains 130 nucleotides.

The length of these regions for recombination is not unimportant. Previous data (Bandyopadhyay, P.K. *et al*. Proc. Natl. Acad. Sci. U.S.A., (1984) *81*, 3476-3480; Rubnitz, J. and Subramani, S. (1984) *supra*) demonstrate that a 10-fold reduction in recombination frequency may occur when sequence homology is reduced from 214 to 163 base pairs. Furthermore, sufficient recombination events should occur within the electroporated cells to ensure that the generated viral phenotype is a reliable reflection of the quasi-species present in the treated HIV-positive patient. Optimisation of recombinant events can first be achieved by adjusting the ratio of linearised proviral vector to RT-PCR fragment that is used for electroporation of the target cells. The standard method therefore,with typical results of outcome, has previously been described by Kellam, P. and Larder, B.A. (1994 *supra*). As a consequence, it was decided to increase the initial input of about 2μg of PCR product (with 10μg of vector) to about 5μg or more. The result thereof was reflected in a faster appearance of visible virus growth (cytopathogenic effect) in the culture of transfected cells.

23

Another option for optimisation of recombination events would be the design of primers resulting in longer recombination sequences.

Nevertheless, the real input in the transfection reaction always depends on the yield after PCR. Some samples have a high yield and as a result there will be a higher input of amplified material in the transfection reaction (with better results on efficiency of recombination). However, despite a lower recombination efficiency, samples having a low yield can also be transfected and will result in viable virus with a reliable reflection of the virus population.

Example 3

Alternative primers for RT-PCR of the ProRT sequence:

New primers (A-D) have been designed relative to those used in Example 2 and should result in longer recombination sequences at both 5' and 3' end of the ProRT region. Two primers were designed at both the 5' and 3' end of the respective region to allow nested PCR. As indicated in Figs. 3 and 4 the direct repeat present at the 5' end of the ProRT region was taken into account when designing the respective primers. The new primers are as follows:

```
A  PRTO-5  : 5'-GCCCCTAGGA-AAAAGGGCTG-TTGG  (SEQ ID NO: 3)
B  PRTI-5   : 5'-TGAAAGATTG-TACTGAGAGA-CAGG  (SEQ ID NO: 4)
C  PRTI-3   : 5'-GATATTTCTC-ATGTTCATCT-TGGG   (SEQ ID NO: 5)
D  PRTO-3  : 5'-AGGTGGCAGG-TTAAAATCAC-TAGC  (SEQ ID NO: 6)
```

Example 4

Construction of an alternative ΔProRT vector:

As mentioned above, construction of an alternative ProRT deleted vector can be achieved by oligonucleotide-mediated mutagenesis. However, it is also possible to enlarge the ProRT deletion from the current 3'-end to the next KpnI site in the RT gene (40 base pairs further

downstream). Ligation of a Klenow-treated KpnI site to a Klenow-treated BstEII site will restore the initial BstEII recognition sequence. As such, this alternative vector behaves similarly to the pGMT3-ΔPRT vector described in Example 2, but has a slightly larger RT deletion.

Example 5

An HIV infected patient who received AZT from December 1989 until an undocumented later date, and switched to a combined chemotherapy of AZT + 3TC (1:1) from February 1994 until October 1995 donated plasma whose susceptibility to a number of RT inhibitors was determined according to the above described protocol of Example 1. Recombinant wild type HIV strain recIIIB was used in said protocol as a reference HIV virus. Table 1 shows the IC_{50} values (μM) measured and the ratio of said values. The Antivirogram is shown in Fig. 5.

Table 1

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
loviride	0.1	0.12	0.11	0.05	2
tivirapine	0.019	0.018	0.019	0.01	1.5
AZT	0.001	0.002	0.002	0.004	0.4
3TC	31.6	100	65.8	0.56	118
d4T	0.07	0.49	0.06	0.12	0.5
ddI	2.0	0.8	1.4	2.83	0.5
ddC	0.2	0.2	0.2	0.38	0.5
AZT+3TC (1:1)	0.001	0.001	0.001	0.002	0.5

From these data, one can determine that monotherapy with 3TC is unlikely to benefit this particular patient. Combined therapy of AZT + 3TC (the current therapy), however, is still likely to exert a positive effect.

25

Example 6

A drug-naive HIV infected patient donated plasma whose susceptibility to a number of RT inhibitors was determined according to the above described protocol of Example 1. Recombinant wild type HIV strain recIIIB was used in said protocol as a reference HIV virus. Table 2 shows the IC$_{50}$ values (μM) measured and the ratio of said values. An Antivirogram was prepared and shown in Fig. 6.

Table 2

Drug	Anti-HIV-1 activity IC$_{50}$ (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
3TC	1.81	2.02	1.91	3.08	1
ddI	3.07	4.47	3.77	8.58	0.4
ddC	1.45	1.47	1.46	2.21	1
AZT	0.04	0.05	0.05	0.06	1
d4T	1.31	0.97	1.14	1.74	1
AZT+3TC (1:1)	0.05	0.04	0.05	0.02	3
DDC+D4T (1:1)	0.62	0.44	0.53	0.77	1
3TC+d4T (1:1)	0.42	0.44	0.43	1.11	0.4

From these data one can determine that the patient is infected with HIV strains closely resembling the wild type HIV. None of the drug regimens is to be excluded, so chemotherapy can be initiated with a drug such as AZT having a positive track record.

Example 7

A drug-naive HIV-infected patient donated plasma whose susceptibility to a number of RT inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain

26

recIIIB was used as a reference HIV virus. Table 3 shows the IC_{50} values (µM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 7A. An Antivirogram was also prepared and is shown in Fig. 7B.

5

Table 3

Drug	Anti-HIV-1 activity IC_{50} (µM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	0.052	0.050	0.051	0.023	2
3TC	2.173	ND	2.173	1.381	2
ddI	0.475	0.429	0.452	0.648	0.7
ddC	1.042	1.389	1.216	1.616	0.8
d4T	1.142	1.657	1.399	1.368	1
loviride	0.035	0.025	0.030	0.024	1
tivirapine	0.042	0.049	0.046	0.021	2

From these data one can determine that the patient is infected with a HIV strain closely resembling the wild type HIV. None of the drug regimens is to be excluded, so chemotherapy can be initiated with a drug
10   such as AZT, 3TC or others having a positive track record.

Example 8

An HIV-infected patient with a therapy history including AZT, 3TC and loviride donated plasma whose susceptibility to a number of RT inhibitors was determined according to the protocol set out in Example 1.
15   Recombinant wild type HIV strain recIIIB was used as a reference HIV virus. Table 4 shows the IC_{50} values (µM) measured and the ratio of said values. A bar graph showing relative change in drug susceptability is shown in Fig. 8A. An Antivirogram was also prepared and is shown in Fig. 8B.

27

Table 4

	Anti-HIV-1 activity IC$_{50}$ (µM)				
Drug	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	18.264	22.251	20.257	0.084	241
3TC	>100.000	>100.000	>100.000	6.304	> 16
ddI	26.861	15.435	21.148	1.586	13
ddC	9.290	8.506	8.898	1.931	5
d4T	7.500	7.097	7.298	5.465	1
loviride	>100.000	>100.000	>100.000	0.037	> 2717
tivirapine	1.626	1.604	1.615	0.021	78

From this data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibility towards most of the nucleoside and non-nucleoside antiretroviral drugs examined. Therapy can still be initiated with D4T or DDC. The possibility of including protease-inhibitors into the therapy can be considered.

Example 9

An HIV-infected patient with a therapy history including multiple nucleoside analogue RT-inhibitors donated plasma whose susceptibility to a number of RT inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIB was used as a reference HIV virus. Table 5 shows the IC$_{50}$ values (µM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 9A. An Antivirogram was also prepared and is shown in Fig. 9B.

28

Table 5

| Drug | Anti-HIV-1 activity IC$_{50}$ (µM) | | | | |
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	> 100.000	ND	> 100.000	0.291	> 344
3TC	> 100.000	> 100.000	> 100.000	16.670	> 6
ddI	> 100.000	> 100.000	> 100.000	4.757	> 21
ddC	60.079	73.049	66.564	3.444	19
d4T	> 100.000	> 100.000	> 100.000	12.030	> 8
loviride	0.064	0.058	0.061	0.065	0.9
tivirapine	0.052	0.043	0.048	0.042	1

From this data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibility towards all nucleoside analogue antiretroviral drugs. Non-nucleoside antiretroviral drugs should not be excluded from therapy. Here also, the possibility of including protease inhibitors into the therapy can be considered.

Example 10

A drug-naive HIV-infected patient donated plasma whose susceptibility to a number of RT inhibitors and protease inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIB was used as a reference HIV virus. Table 6 shows the IC$_{50}$ values (µM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 10A. An Antivirogram was also prepared and is shown in Fig. 10B.

29

Table 6

Drug	Anti-HIV-1 activity IC_{50} (µM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio(1)/(2)
AZT	0.019	0.019	0.019	0.041	0.5
3TC	1.525	1.718	1.622	4.608	0.4
saquinavir	0.003	0.003	0.003	0.006	0.4
ritonavir	0.022	0.017	0.019	0.033	0.6
indinavir	0.013	0.013	0.013	0.016	0.8

From these data one can determine that the patient is infected with HIV strains closely resembling the wild type HIV. None of the drug
5   regimens is to be excluded, so that chemotherapy can be initiated with a drug such as AZT, 3TC or others having a positive track record.

Example 11

An HIV infected patient with a therapy history including RT and protease inhibitors donated plasma whose susceptibility to a number of
10   RT inhibitors and protease inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIb was used as a reference HIV virus. Table 7 shows the IC_{50} values (µM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 11A. An
15   Antivirogram was also prepared and is shown in Fig. 11B.

Table 7

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	ND	0.015	0.015	0.047	0.3
3TC	> 100.000	93.962	96.981	5.178	19
saquinavir	0.014	0.015	0.014	0.012	1
ritonavir	1.198	1.739	1.468	0.062	24
indinavir	0.229	0.416	0.323	0.027	12

From these data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibilty towards the RT-inhibitor 3TC and protease inhibitors indinavir and ritonavir. Accordingly, chemotherapy can be adjusted with drugs such as AZT or saquinavir having a positive track record.

Example 12

An HIV infected patient with a therapy history including RT and protease inhibitors donated plasma whose susceptibility to a number of RT inhibitors and protease inhibitors was determined according to the protocol set out in Example 1. Table 8 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 12A. An Antivirogram was also prepared and is shown in Fig. 12B.

Table 8

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	3.833	3.355	3.594	0.041	88
3TC	>100.000	>100.000	>100.000	4.608	22
saquinavir	0.350	0.352	0.351	0.006	56
ritonavir	1.610	1.530	1.570	0.033	47
indinavir	0.124	ND	0.124	0.016	8

From these data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibility towards RT-inhibitors 3TC and AZT and protease inhibitors indinavir, ritonavir and saquinavir.

Example 13

Comparison of Phenotyping relative to Genotyping

Plasma samples were obtained from HIV-infected individuals who had been receiving non-nucleoside RT inhibitor (NNRTI) long-term monotherapy. HIV-RNA was extracted, reverse-transcribed and amplified as described in Example 1. Starting from outer PCR material of positive samples, the first 785 nucleotides of the RT gene were amplified and this material was further used for genotyping.

Briefly, the 785 nucleotide fragment was subjected to cycle-sequencing reactions using the ThermoSequenase (ThermoSequenase is a Trade Mark) fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP from Amersham (cat# RPN2438). Four sequencing primers, chosen to allow for sequence determination in both directions from nucleotide 27 to nucleotide 681 of the RT gene, were used for each sample. The reactions were analysed on an ALF (ALF is a Trade Mark) automatic sequencer (Pharmacia). The generated sequences were exported to a Power Macintosh and further analysed with the

GeneWorks 2.5 software (Oxford Molecular Group Inc.). Resulting amino acid sequences were compared with the corresponding sequence of the laboratory HIV-1 clone HXB2D and resistance-associated mutations identified in patient material. The results are shown in Table

5   9 where the one-letter amino acid code is used.

Table 9

P	RESISTANCE ASSOCIATED MUTATIONS													FOLD RESISTANCE TO			
	M 41	D 67	K 70	A 98	K 101	K 103	V 108	E 138	Y 181	M 184	G 190	T 215	K 219	AZT	3TC	NNRTI 1	NNRTI 2
1						N								1	1	56	437
2						S								1	0.4	102	53
3						N								0.4	1	36	87
4														0.3	0.1	1	0.4
5														1	0.4	4	3
6						N								1	0.3	103	245
7						S								1	0.04	112	57
8						N								1	1	30	81
9									C					2	1	>1432	12
10						N								0.4	0.1	53	172
11	L	N	R			N				V		F	Q	94	>8	321	669
12	L											Y		28	2	1	3
13				E	K/N			A			G/A		Not Det	1	1	>1466	455
14						N								1	1	93	349
15						N								2	1	>2424	449
16						N				V				1	>8	21	115
17						N								1	1	29	102
18						S								1	1	95	181
19						N								1	1	78	260
20				G										0.4	1	22	25
21						N								1	0.4	47	68
22							I							1	0.4	3	3
23						N								0.2	0.2	9	9
24				Q										1	1	7	59

P = Patient

The top row of Table 9 shows the aminoacids (AA) found in the wild type sequence and their position. Amino acids changes at these positions are shown for each patient in the following rows. Only the positions at which changes were observed in patient material are shown. The right part of Table 9 presents the fold resistance to different RT inhibitors as determined by the method according to the invention for each of the patients' samples. NNRTI 1 is the non-nucleoside RT inhibitor that was administered to the patients. NNRTI 2 is another non-nucleoside RT inhibitor for which cross-resistance with the first one was observed to some extent.

The genotyping results regarding nucleoside analog RT inhbitors resistance are as follows :

- M41L, D67N, K70R, T215F/Y and K219Q/E are AZT resistance-associated mutations (Larder, B. and Kemp, S. (1989) Science 246, 1155-1158; Kellam, P. et al. (1992) PNAS 89, 1934-1938). Their presence, individually or in different combinations, in the genome of HIV isolated from patient material correlates with the phenotypic resistance as determined by the Antivirogram generated (patients 11 and 12).

- The same applies to resistance to 3TC associated with the M184V mutation (Tisdale, M. et al. (1993) PNAS 90, 5653-5656) which is observed only in the patients which show phenotypic resistance to the drug (patients 11 and 16).

The genotyping results regarding NNRTIs resistance are as follows :

- Three patients (3, 4 and 12) have no NNRTI resistance-associated mutation and are phenotypically sensitive to the drug.

- Most of the patients who show phenotypic resistance to the NNRTIs have a NNRTI resistance-associated mutation at position 103 (K103N/S).

34

- One patient (9) has the Y181C NNRTI resistance-associated mutation and shows a high phenotypic resistance (>1432 fold) to NNRTI 1.

5

10

- Patient 13 has several NNRTI resistance-associated mutations (K101E, K103N partially and G190A partially). This patient also shows a high phenotypic resistance (>1466 fold) to NNRTI 1. The E138A mutation observed in this sample is not associated so far with resistance. However, another mutation at this same position, i.e. E138K, has been demonstrated to play an important role in resistance to the TSAO compounds (Balzarini, J. *et al.* (1993) PNAS *90*, 6952-9656). The role of the E138A mutation still needs to be assessed.

- Patient 20 has the A98G NNRTI resistance-associated mutation and shows phenotypic resistance to the tested NNRTIs.

15

- Patient 22 has the V108I NNRTI resistance-associated mutation but does not show any phenotypic resistance to the tested NNRTIs.

- Patient 24 shows no NNRTI resistance-associated mutation (the K101Q mutation is found in several HIV-1 wild type genomes) but is phenotypically resistant to the tested NNRTIs.

20

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: VIRCO N.V.
        (B) STREET: Drie Eikenstraat 661
        (C) CITY: Edegem
        (E) COUNTRY: Belgium
        (F) POSTAL CODE (ZIP): B-2650

        (A) NAME: DE BETHUNE, Marie-Pierre
        (B) STREET: Tweeleeuwenstraat 15
        (C) CITY: Everburg
        (E) COUNTRY: Belgium
        (F) POSTAL CODE (ZIP): B-3078

        (A) NAME: HERTOGS, Kurt
        (B) STREET: Sint Vincentiusstraat 53
        (C) CITY: Antwerpen
        (E) COUNTRY: Belgium
        (F) POSTAL CODE (ZIP): B-2018

        (A) NAME: PAUWELS, Rudi
        (B) STREET: Damstraat 166
        (C) CITY: Weerde
        (E) COUNTRY: Belgium
        (F) POSTAL CODE (ZIP): B-1982

    (ii) TITLE OF INVENTION: Method of managing the chemotherapy of
        patients who are HIV positive based on the phenotypic drug
        sensitivity of human HIV strains

    (iii) NUMBER OF SEQUENCES: 6

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: EP 96200175.6
        (B) FILING DATE: 26-JAN-1996

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
    (A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CATTGCTCTC CAATTACTGT GATATTTCTC ATG   33

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Human immunodeficiency virus type 1

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGGAAGATCT GGCCTTCCTA CAAGGG   26

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Human immunodeficiency virus type 1

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCCCTAGGA AAAAGGGCTG TTGG   24

37

(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Human immunodeficiency virus type 1

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGAAAGATTG TACTGAGAGA CAGG   24

(2) INFORMATION FOR SEQ ID NO: 5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Human immunodeficiency virus type 1

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATATTTCTC ATGTTCATCT TGGG   24

(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Human immunodeficiency virus type 1

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGTGGCAGG TTAAAATCAC TAGC   24

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMBP-COLLECTION
Page 1 of Form BCCM/LMBP/BP/4/96-07   Receipt in the case of an original deposit

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Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the International Depositary Authority BCCM/LMBP identified at the bottom of next page

International Form BCCM/LMBP/BP/4/96-07

To :   Name of the depositor : VIRCO nv.

          Address   : Drie Eikenstraat. 661
                                     2650 Edegem

I.   Identification of the microorganism:

   I 1   Identification reference given by the depositor:

   > pGEMT3ΔPRT

   I.2   Accession number given by the International Depositary Authority:

   > LMBP3590

SUBSTITUTE SHEET (RULE 26)

39

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
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Page 2 of Form BCCM/LMBP/BP/4/96-07   Receipt in the case of an original deposit

_____

II.   Scientific description and/or proposed taxonomic designation

        The microorganism identified under I above was accompanied by:

        (mark with a cross the applicable box(es)):

        ☒ .   a scientific description

        ☐   a proposed taxonomic designation

III.   Receipt and acceptance

        This International Depositary Authority accepts the microorganism identified under I
        above, which was received by it on (date of original deposit) : November 08, 1996

IV   International Depositary Authority

        Belgian Coordinated Collections of Microorganisms (BCCM)
        Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)
        Universiteit Gent
        K.L. Ledeganckstraat 35
        B-9000 Gent, Belgium

        Signature(s) of person(s) having the power to represent the International Depositary
        Authority or of authorized official(s):

        Date   : November 19, 1996   Lic Martine Vanhoucke

SUBSTITUTE SHEET (RULE 26)

<u>Claims</u>: -

1.   A method of managing HIV chemotherapy of patients who are HIV positive, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses, assessing the phenotypic sensitivity of said chimeric viruses to an inhibitor of said enzyme encoded by the pol gene of HIV and assigning a value thereto, constructing a data set comprising said value for chimeric virus sensitivity and the corresponding value for a chimeric wild-type strain of HIV, repeating the sensitivity assessment for at least two further inhibitors and thereby constructing at least three such data sets in total, representing said data sets in two dimensional or three dimensional graphical form such that the difference between the chimeric and wild-type sensitivities in the case of each data set provides a visual measure of the resistance of the chimeric stock to treatment by the inhibitor in question, and selecting the optimum inhibitor(s) on the basis of the graphical representation of the resistances so measured.

2.   A method of managing HIV chemotherapy according to Claim 1, wherein the data sets are represented on a polygonal or quasi-circular graph comprising:

     (a)   a plurality of normalised axes extending radially from an origin, each axis corresponding to one data set or inhibitor or combination thereof;

     (b)   the axes being normalised such that the sensitivity values for wild-type HIV for the various inhibitors are equal on each axis, the data points for wild-type HIV being optionally represented and connected to form a regular polygon whose vertices lie on the axes and whose center is defined by the origin;

(c)   on each axis a data point representing the sensitivity value of the chimeric HIV stock against the inhibitor corresponding to said axis is plotted, the chimeric data points being optionally connected to form a regular or irregular polygon the shape of which represents the resistance of the chimeric stock to a range of inhibitors.

3.   A method according to Claim 2, wherein each axis has a logarithmic scale.

4.   A method according to Claim 2 or 3, wherein eccentric data points in the chimeric polygon, if represented, identify inhibitors whose usefulness can be assumed to be of little or no benefit to the patient, while data points lying within, on or close outside the wild-type polygon identify inhibitors whose usefulness can be assumed to be of substantial benefit to the patient.

5.   A method according to any preceding claim, wherein each of said three or more data sets further comprises a value for worst-case measurable resistance for the inhibitor in question, said worst case values being represented on said graphical representations such that the data point for the chimeric stock can be compared both to wild-type and to worst-case HIV, thereby providing an assessment of the relative value of the inhibitor in a particular case.

6.   A method of managing HIV chemotherapy of patients who are HIV positive, which comprises the steps of:

(a)   periodically assessing the phenotypic sensitivity of a patient's HIV strains according to any one of Claims 1-5;

(b)   maintaining the chemotherapy with the selected inhibitor while the patient's HIV strains remain susceptible to the selected chemotherapy;

(c)   selecting a different inhibitor if and when the susceptibility of the original inhibitor decreases.

7.   A clinical management device for use in the management of chemotherapy of patients who are HIV positive, said device bearing a graphical representation of a plurality of data sets as defined in Claim 1.

8.   A method according to any one of Claims 1-6, wherein the phenotypic sensitivity of said chimeric viruses to inhibitors of at least two enzymes encoded by the pol gene of HIV is simultaneously assessed.

9.   A method of determining the phenotypic drug sensitivity of individual HIV strains in a patient to inhibitors of at least two enzymes encoded by the pol gene of HIV, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses and assessing the phenotypic sensitivity of said chimeric viruses to inhibitors of said enzymes encoded by the pol gene of HIV.

10.   A method according to any one of Claims 1-6, 8 and 9, wherein said sequence from the pol gene is isolated from a sample of a biological material obtained from the patient whose phenotypic drug sensitivity is being determined.

11.   A method according to Claim 10, wherein said biological material is selected from plasma, serum or a cell-free body fluid selected from semen and vaginal fluid.

12.   A method according to Claim 10, wherein the biological material is whole blood to which an RNA stabiliser has been added.

13.   A method according to Claim 10, wherein the biological material is tissue material selected from brain tissue or lymph nodal tissue.

14.   A method according to any one of Claims 9-13, wherein the at least two enzymes are selected from HIV RT, protease and integrase.

15.   A method according to any one of Claims 1-6 and 8-14, wherein the cell line susceptible to infection by HIV is a CD4$^+$ T-cell line.

16.   A method according to Claim 15, wherein the CD4$^+$ T-cell line is the MT4 cell line or the HeLa CD4$^+$ cell line.

17.   A method according to any one of Claims 1-6 and 8-16, wherein the desired region of the patient's pol gene is reverse transcribed using a specific downstream primer.

18.   A method according to Claim 17, wherein the sequence to be reverse transcribed is that coding for reverse transcriptase and protease.

19.   A method according to Claim 18, wherein the downstream primer is OUT3 : 5'-CAT TGC TCT CCA ATT ACT GTG ATA TTT CTC ATG-3' (SEQ ID NO: 1).

20.   A method according to any one of Claims 17-19, wherein the product of reverse transcription is amplified using a nested PCR technique.

21.   A method according to any one of Claims 1-6 and 8-20, wherein the HIV-DNA construct is one from which the RT and protease genes are deleted and is the plasmid pGEMT3-ΔPRT as deposited at the Belgian Coordinated Collections of Microorganisms-BCCM LMBP-Collection on November 8, 1996 under the number LMBP3590.

22. A method according to any one of Claims 1-6 and 8-21, wherein the transfection is achieved by electroporation.

23. A method according to any one of Claims 1-6 and 8-21, wherein the transfection is achieved by the use of cationic lipids.

5   24. A method according to any one of Claims 1-6 and 8-23, wherein the phenotypic drug sensitivity of the chimeric viruses to different RT, protease and integrase inhibitors is assessed in an automated cellular-based assay.

25. A method according to any one of Claims 1-6 and 8-24, 10   wherein the phenotypic drug sensitivity of the chimeric viruses and of the wild HIV strain to one or more RT, protease or integrase inhibitor(s) is expressed as an inhibitory concentration (IC value).

26. A method according to any one of Claims 1-6 and 8-25, wherein RT inhibitors are selected from nucleoside RT inhibitors such as 15   AZT, ddI, ddC, 3TC, d4T, non-nucleoside RT inhibitors such as loviride, nevirapine and tivirapine, protease inhibitors such as saquinavir, indinavir and ritonavir and integrase inhibitors such as caffeic acid phenylethyl ester (CAPE).

27. A method of managing HIV chemotherapy of patients who 20   are HIV positive, substantially as hereinbefore described and exemplified.

28. A clinical management device, substantially as hereinbefore described and exemplified with particular reference to and as illustrated in Figs. 5-12 of the accompanying drawings.

25   29. A method of determining the phenotypic drug sensitivity of individual HIV strains in a patient to inhibitors of at least two enzymes encoded by the pol gene of HIV, substantially as hereinbefore described and exemplified.

1/18

Fig. 1

STEP 1

Digestion of pHIVΔRTBstEII with Ahdl
Treatment with DNA pol I (Klenow fragment)
Digestion with Apal
Purify ~270 bp Apal-Ahdl fragment and
subclone into pGEM7zf(-) opened with
Apal/Smal

STEP 2

Subclone Xbal fragment
on pGEM9zf(-) to make
Apal site unique

pHIVΔRTBstEII
pIBI20-derivative

pGEM7-T1

pGEM9-T2

STEP 3

Substitute ~750 bp Apal-BstEII fragment of
pGEM9-T2 (BstEII-ends Klenow treated)
with ~270 bp Apal-XmaIID fragment of
pGEM7-T1 (Xmal-ends Klenow treated)

pGEM9-T3

▬ Human genomic sequences flanking HIV provirus
▨ ΔRTBstEII
▨ Protease

2/18

Fig. 2

STEP 1

5'-GACNNN/NNGTC (Ahdl recognition sequence and cleavage site)

5'-GACCCC/TCGTC (Ahdl site at the beginning of the protease coding region)

> Ahdl cleavage
> Removal of 1-nucleotide 3'-overhang
> by treatment with DNA polymerase I
> (Klenow fragment)

5'-GACCC ◄─────────────────────────► 5'-CCC/GGG (Smal)

> ligation of blunt ends
> restores the Smal site
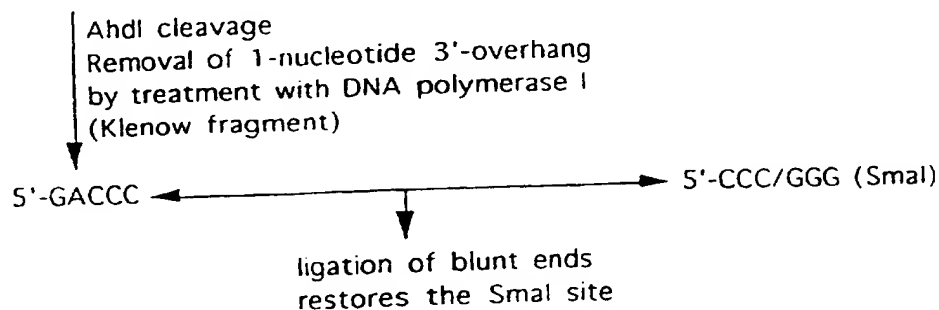
STEP 3

5'-GAC/CCGGG

5'-G/GTNACC (BstEll)
5'-G/GTGACC (BstEll in ΔRT-clone)

> The restored Smal recognition site is cleaved
> by Xmal (creating a 4 nucleotide 5'-overhang)
> and converted to a blunt end by treatment with
> DNA polymerase I (Klenow fragment)
> Similarly, the BstEll-digested recipient vector
> pGEM9-T2 is treated with DNA pol I (Klenow
> fragment) prior to digestion with Apal
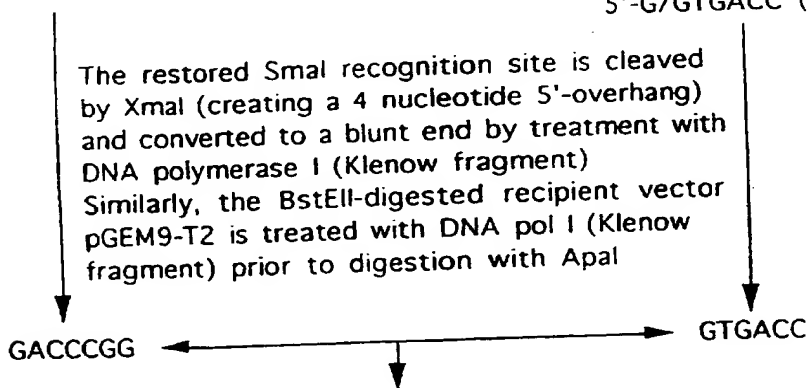
GACCCGG ◄─────────────────────────► GTGACC

> GA<u>CCC</u>gggtgACC (underlined codon is P9 in protease)
> There is both a Smal/Xmal and BstEll at the ΔProRT-junction
> "Foreign sequences" at the ΔProRT-junction are represented by
> lower case letters

● ●

Fig. 3
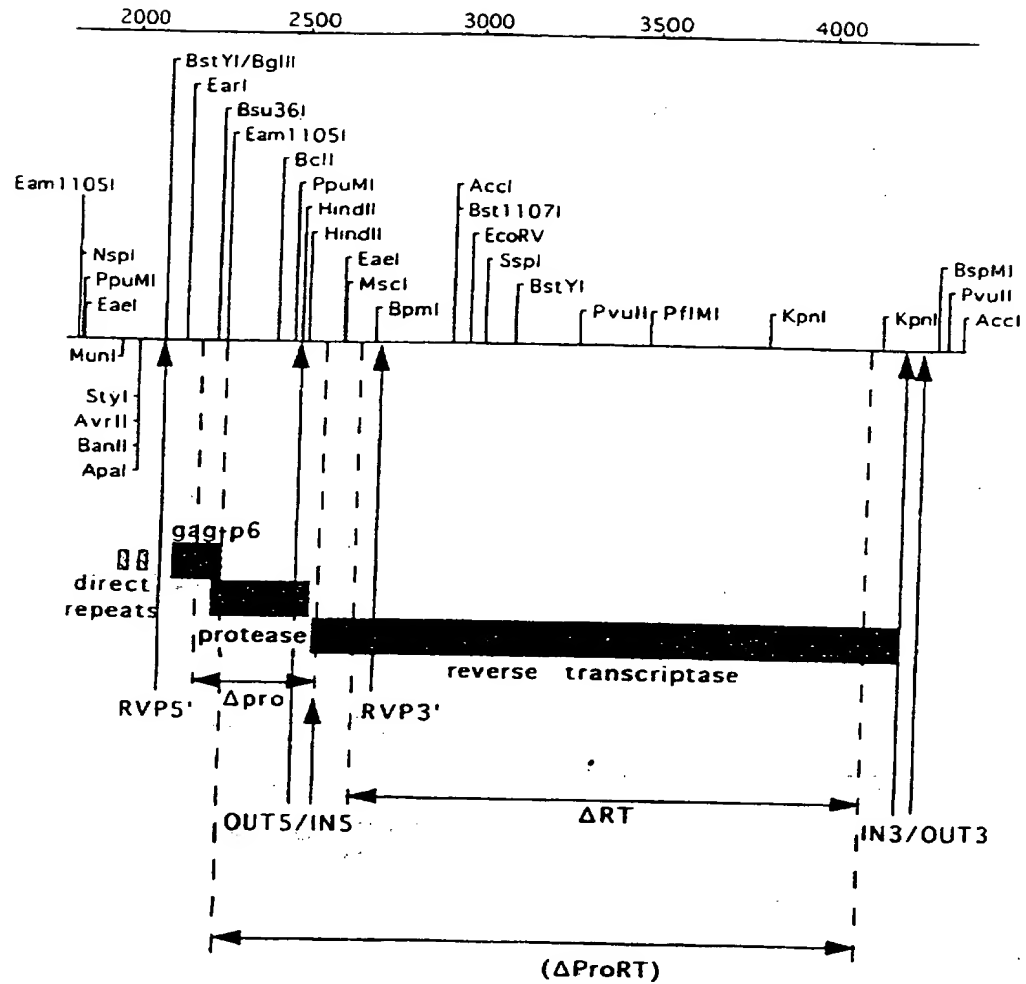
HXB2 (sequence range: 1800 to 4400)

Fig. 4A

```
                                                  >NspI
                                                   |
                                         >Eam1105I |
                                            |      |
       1800        1810        1820        1830    |  |1840
        •           •           •           •      |  |  •
      GGA CCA GCG GCT ACA CTA GAA GAA ATG ATG ACA GCA TGT CAG
       G   P   A   A   T   L   E   E   M   M   T   A   C   Q
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___


              >PpuMI     >EaeI
                |          |
          1850  |          |  1860        1870        1880
           •    |          |   •           •           •
      GGA GTA GGA GGA CCC GGC CAT AAG GCA AGA GTT TTG GCT GAA
       G   V   G   G   P   G   H   K   A   R   V   L   A   E
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___


              1890        1900        1910        1920
               •           •           •           •
      GCA ATG AGC CAA GTA ACA AAT TCA GCT ACC ATA ATG ATG CAG
       A   M   S   Q   V   T   N   S   A   T   I   M   M   Q
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___

                                                           >MunI
                                                             |
                                                             |
          1930        1940        1950        1960           |
           •           •           •           •             |
      AGA GGC AAT TTT AGG AAC CAA AGA AAG ATT GTT AAG TGT TTC
       R   G   N   F   R   N   Q   R   K   I   V   K   C   F
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___

                                                           >BanII
                                                             |
                                                           >ApaI
                                                             |
                                                             |
          1970        1980        1990        2000           |
           •           •           •           •             |
      AAT TGT GGC AAA GAA GGG CAC ACA GCC AGA AAT TGC AGG GCC
       N   C   G   K   E   G   H   T   A   R   N   C   R   A
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
      <---------------direct repeat#1 ----------->

  >AvrII
    |
  >StyI
    |
  2010        2020        2030        2040        2050
    •           •           •           •           •
      CCT AGG AAA AAG GGC TGT TGG AAA TGT GGA AAG GAA GGA CAC
       P   R   K   K   G   C   W   K   C   G   K   E   G   H
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
                                   <-----direct repeat#2 --

          2060        2070        2080        2090
           •           •           •           •
      CAA ATG AAA GAT TGT ACT GAG AGA CAG GCT AAT TTT TTA GGG
       Q   M   K   D   C   T   E   R   Q   A   N   F   L   G
      ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
      ------------------->                           <--
```

5/18

Fig. 4B

```
>BglII
  :
>BstYJ
  |
  |     2100           2110           2120           2130
  |      •             •              •              •
  AAG ATC TGG CCT TCC TAC AAG GGA AGG CCA GGG AAT TTT CTT
   K   I   W   P   S   Y   K   G   R   P   G   N   F   L
  ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
                                                        L
                                                       ___

  ---- RVP5 -------------------->

                               >EarI
                                |
      2140           2150      2160|        2170
       •             •          • |         •
  CAG AGC AGA CCA GAG CCA ACA GCC CCA CCA GAA GAG AGC TTC
   Q   S   R   P   E   P   T   A   P   P   E   E   S   F
  ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
   Q   S   R   P   E   P   T   A   P   P   E   E   S   F
  ___b___b___b___b____GAG P6 (52 AA)_b___b___b___b___b___

  2180           2190           2200           2210
   •             •              •              •
  AGG TCT GGG GTA GAG ACA ACA ACT CCC CCT CAG AAG CAG GAG
   R   S   G   V   E   T   T   T   P   P   Q   K   Q   E
  ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
   R   S   G   V   E   T   T   T   P   P   Q   K   Q   E
  ___b___b___b___b____GAG P6 (52 AA)_b___b___b___b___b___

                                          >Bsu36I
                                             |
  2220           2230           2240      2250  |      2260
   •             •              •          •    |       •
  CCG ATA GAC AAG GAA CTG TAT CCT TTA ACT TCC CTC AGG TCA
   P   I   D   K   E   L   Y   P   L   T   S   L   R   S
  ___a___a___a___a___GAG POLYPROTEIN_a___a___a___a___a___
   P   I   D   K   E   L   Y   P   L   T   S   L   R   S
  ___b___b___b___b____GAG P6 (52 AA)_b___b___b___b___b___
                                              P   Q   V
                                           _c___c___c___

      |--->ΔPro

                   >Eam1105I
                        |
      2270           2280           2290           2300
       •             |  •              •              •
  CTC TTT GGC AAC GAC CCC TCG TCA CAA TAA AG ATA GGG GGG
   L   F   G   N   D   P   S   S   Q   •
  ___a___a___GAG POLYPROTEIN_a___a___a___
   L   F   G   N   D   P   S   S   Q
  ___b___b__GAG P6 (52 AA)___b___b___
   T   L   W   Q   R   P   L   V   T   I   K   I   G   G
  ___c___c___c___c___c__PROTEASE_c___c___c___c___c___c___
                           |-->ΔProRT (Tibotec)

      2310           2320           2330           2340
       •             •              •              •
  CAA CTA AAG GAA GCT CTA TTA GAT ACA GGA GCA GAT GAT ACA
   Q   L   K   E   A   L   L   D   T   G   A   D   D   T
  __c___c___c___c___c_ PROTEASE___c___c___c___c___c___
```

6/18

Fig. 4C

```
       2550          2360          2370          2380
GTA TTA GAA GAA ATG AGT TTG CCA GGA AGA TGG AAA CCA AAA
 V   L   E   E   M   S   L   P   G   R   W   K   P   K
___ c___c___c___c___c    PROTEASE____c   c___c__ c___c___

                                                 >BclI
                                                   |
    2390          2400          2410          2420 |
ATG ATA GGG GGA ATT GGA GGT TTT ATC AAA GTA AGA CAG TAT
 M   I   G   G   I   G   G   F   I   K   V   R   Q   Y
___ c___c___c___c___c_ PROTEASE____c   c__ c___c___c__

2430          2440          2450          2460          247C
GAT CAG ATA CTC ATA GAA ATC TGT GGA CAT AAA GCT ATA GGT
 D   Q   I   L   I   E   I   C   G   H   K   A   I   G
___ c___c___c___c___ c   _PROTEASE____c___c   _c___c___c__
                                                        <--

         >PpuMI              >HindII
           |                   |
    2480   |      2490         |2500          2510
ACA GTA TTA GTA GGA CCT ACA CCT GTC AAC ATA ATT GGA AGA
 T   V   L   V   G   P   T   P   V   N   I   I   G   R
___c__ c___c___c_  c___PROTEASE____c___c   _c___c___c__
------- OUT5 --------------------------->

     >HindII
        |
    2520          2530          2540          2550
AAT CTG TTG ACT CAG ATT GGT TGC ACT TTA AAT TTT CCC ATT
 N   L   L   T   Q   I   G   C   T   L   N   F
___c___c___c___c___PROTEASE____c___c___c___c___
                                             P   I
                                            ___d___
                                            <------------

    2560          2570          2580          2590
AGC CCT ATT GAG ACT GTA CCA GTA AAA TTA AAG CCA GGA ATG
 S   P   I   E   T   V   P   V   K   L   K   P   G   M
___d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d__
--- IN5 ----------------------->
     ΔPro --->|

                          >MscI
                            |
                          >EaeI
                          | |
    2600          2610    |2620          2630
GAT GGC CCA AAA GTT AAA CAA TGG CCA TTG ACA GAA GAA AAA
 D   G   P   K   V   K   Q   W   P   L   T   E   E   K
__ d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d___
```

Fig. 4D

```
      2640         2650           2660          2670          2680
       .            .              .             .             .
ATA AAA GCA TTA GTA GAA ATT TGT ACA GAG ATG GAA AAG GAA
 I   K   A   L   V   E   I   C   T   E   M   E   K   E
 __d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d__
                                           |--->ΔRT

                                      >BpmI
                                         |
      2690         2700           | 2710          2720
       .            .             |   .            .
GGG AAA ATT TCA AAA ATT GGG CCT GAA AAT CCA TAC AAT ACT
 G   K   I   S   K   I   G   P   E   N   P   Y   N   T
  d___d___d___ REVERSE TRANSCRIPTASE__d___d___d___d_

      2730         2740           2750          2760
       .            .              .             .
CCA GTA TTT GCC ATA AAG AAA AAA GAC AGT ACT AAA TGG AGA
 P   V   F   A   I   K   K   K   D   S   T   K   W   R
 ___d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d__

      2770         2780           2790          2800
       .            .              .             .
AAA TTA GTA GAT TTC AGA GAA CTT AAT AAG AGA ACT CAA GAC
 K   L   V   D   F   R   E   L   N   K   R   T   Q   D
 ___d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d___

     2810         2820           2830          2840
      .            .              .             .
TTC TGG GAA GTT CAA TTA GGA ATA CCA CAT CCC GCA GGG TTA
 F   W   E   V   Q   L   G   I   P   H   P   A   G   L
 __ d___d___d___REVERSE TRANSCRIPTASE__d___d___d___d___

2850         2860           2870          2880          2890
 .            .              .             .             .
AAA AAG AAA AAA TCA GTA ACA GTA CTG GAT GTG GGT GAT GCA
 K   K   K   K   S   V   T   V   L   D   V   G   D   A
 ___d___d___d___ REVERSE TRANSCRIPTASE__d___d___d___d___

                                          >Bst1107I
                                             |
                                            >AccI
                                             ||
      2900         2910           2920       ||2930
       .            .              .         || .
TAT TTT TCA GTT CCC TTA GAT GAA GAC TTC AGG AAG TAT ACT
 Y   F   S   V   P   L   D   E   D   F   R   K   Y   T
 ___d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d___

      2940         2950           2960          2970
       .            .              .             .
GCA TTT ACC ATA CCT AGT ATA AAC AAT GAG ACA CCA GGG ATT
 A   F   T   I   P   S   I   N   N   E   T   P   G   I
 ___d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d__

 >EcoRV
    |
    |
    2980         2990           3000          3010
    | .           .              .             .
AGA TAT CAG TAC AAT GTG CTT CCA CAG GGA TGG AAA GGA TCA
 R   Y   Q   Y   N   V   L   P   Q   G   W   K   G   S
 ___d___d___d____REVERSE TRANSCRIPTASE__d___d___d___d___
```

8/18

Fig. 4E

```
                >SspI
                 :
     3020        :      3030          3040              3050
      •          :       •             •                •
     CCA GCA ATA TTC CAA AGT AGC ATG ACA AAA ATC TTA GAG CCT
      P   A   I   F   Q   S   S   M   T   K   I   L   E   P
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

     3060          3070          3080          3090          3100
       •            •             •             •             •
     TTT AGA AAA CAA AAT CCA GAC ATA GTT ATC TAT CAA TAC ATG
      F   R   K   Q   N   P   D   I   V   I   Y   Q   Y   M
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

                        >BstYI
                          |
          3110            | 3120         3130          3140
            •             |  •            •             •
     GAT GAT TTG TAT GTA GGA TCT GAC TTA GAA ATA GGG CAG CAT
      D   D   L   Y   V   G   S   D   L   E   I   G   Q   H
      _ d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

          3150          3160          3170          3180
            •             •             •             •
     AGA ACA AAA ATA GAG GAG CTG AGA CAA CAT CTG TTG AGG TGG
      R   T   K   I   E   E   L   R   Q   H   L   L   R   W
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

          3190          3200          3210          3220
            •             •             •             •
     GGA CTT ACC ACA CCA GAC AAA AAA CAT CAG AAA GAA CCT CCA
      G   L   T   T   P   D   K   K   H   Q   K   E   P   P
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

     3230          3240          3250          3260
       •             •             •             •
     TTC CTT TGG ATG GGT TAT GAA CTC CAT CCT GAT AAA TGG ACA
      F   L   W   M   G   Y   E   L   H   P   D   K   W   T
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

                                      >PvuII
                                        |
     3270          3280          3290   | 3300          3310
       •             •             •    |  •             •
     GTA CAG CCT ATA GTG CTG CCA GAA AAA GAC AGC TGG ACT GTC
      V   Q   P   I   V   L   P   E   K   D   S   W   T   V
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

          3320          3330          3340          3350
            •             •             •             •
     AAT GAC ATA CAG AAG TTA GTG GGG AAA TTG AAT TGG GCA AGT
      N   D   I   Q   K   L   V   G   K   L   N   W   A   S
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__

          3360          3370          3380          3390
            •             •             •             •
     CAG ATT TAC CCA GGG ATT AAA GTA AGG CAA TTA TGT AAA CTC
      Q   I   Y   P   G   I   K   V   R   Q   L   C   K   L
      _d__ d__ d____REVERSE TRANSCRIPTASE__d___d___d___d__
```
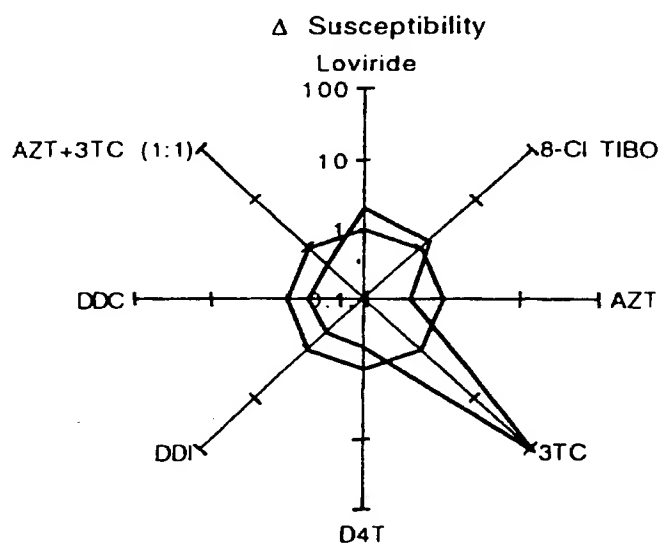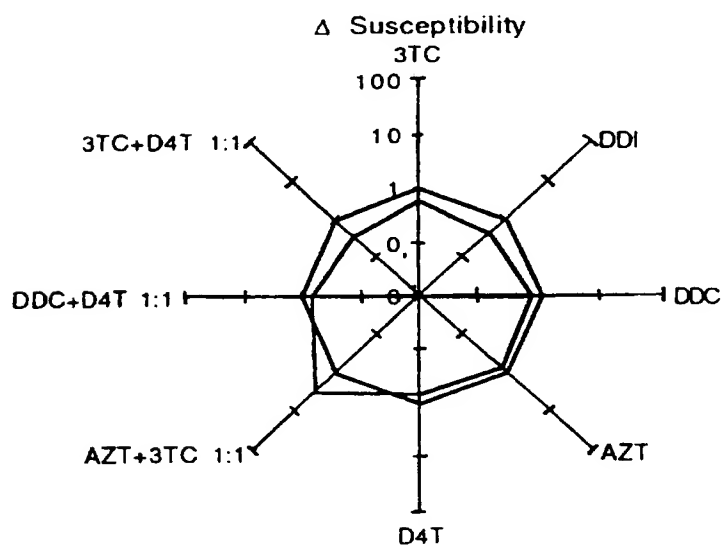
9/18

Fig. 4F

```
         3400           3410          3420          3430
          .             .             .             .
CTT AGA GGA ACC AAA GCA CTA ACA GAA GTA ATA CCA CTA ACA
 L   R   G   T   K   A   L   T   E   V   I   P   L   T
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3440           3450          3460          3470
          .             .             .             .
GAA GAA GCA GAG CTA GAA CTG GCA GAA AAC AGA GAG ATT CTA
 E   E   A   E   L   E   L   A   E   N   R   E   I   L
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

                >PflMI
                   |
3480          3490 |     3500          3510          3520
 .             .   |      .             .             .
AAA GAA CCA GTA CAT GGA GTG TAT TAT GAC CCA TCA AAA GAC
 K   E   P   V   H   G   V   Y   Y   D   P   S   K   D
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3530           3540          3550          3560
          .             .             .             .
TTA ATA GCA GAA ATA CAG AAG CAG GGG CAA GGC CAA TGG ACA
 L   I   A   E   I   Q   K   Q   G   Q   G   Q   W   T
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3570           3580          3590          3600
          .             .             .             .
TAT CAA ATT TAT CAA GAG CCA TTT AAA AAT CTG AAA ACA GGA
 Y   Q   I   Y   Q   E   P   F   K   N   L   K   T   G
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3610           3620          3630          3640
          .             .             .             .
AAA TAT GCA AGA ATG AGG GGT GCC CAC ACT AAT GAT GTA AAA
 K   Y   A   R   M   R   G   A   H   T   N   D   V   K
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3650           3660          3670          3680
          .             .             .             .
CAA TTA ACA GAG GCA GTG CAA AAA ATA ACC ACA GAA AGC ATA
 Q   L   T   E   A   V   Q   K   I   T   T   E   S   I
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

3690          3700          3710          3720          3730
 .             .             .             .             .
GTA ATA TGG GGA AAG ACT CCT AAA TTT AAA CTG CCC ATA CAA
 V   I   W   G   K   T   P   K   F   K   L   P   I   Q
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3740           3750          3760          3770
          .             .             .             .
AAG GAA ACA TGG GAA ACA TGG TGG ACA GAG TAT TGG CAA GCC
 K   E   T   W   E   T   W   W   T   E   Y   W   Q   A
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___

         3780           3790          3800          3810
          .             .             .             .
ACC TGG ATT CCT GAG TGG GAG TTT GTT AAT ACC CCT CCC TTA
 T   W   I   P   E   W   E   F   V   N   T   P   P   L
 __d___d___d____ REVERSE TRANSCRIPTASE__d___d___d___d___
```

● ●

Δ Susceptibility
Loviride

Fig. 5

Δ Susceptibility
3TC

Fig. 6

SUBSTITUTE SHEET (RULE 26)

Fig. 7A

Fig. 7B

14/18

Relative Change in Drug Susceptibility

▨ : Patient Resistance

▢ : Max. Measurable Resistance

⊣ : standard dev.   Normal

Increasing Resistance →

DRUG	Fold Resistance	x 0.1	1	10	100	10³
1) AZT	= 241					
2) 3TC	> 16					
3) DDI	= 13					
4) DDC	= 5					
5) D4T	= 1					
6) LOVIRIDE	> 2717					
7) TIVIRAPINE	= 78					

Fig. 8A

Resistance Level (Log units)

1) AZT
10,000
1,000
100
10

7) TIVIRAPINE

2) 3TC

6) LOVIRIDE

3) DDI

5) D4T

4) DDC

▢ Maximal Measurable Resistance

▨ Patient Sample Actual Resistance

‖ Reference Strain IIIB

Fig. 8B

SUBSTITUTE SHEET (RULE 26)

Fig. 9A

Fig. 9B

Relative Change in Drug Susceptibility

▨ : Patient Resistance

▢ : Max. Measurable Resistance

⊣ : standard dev.   Normal

Increasing Resistance

DRUG	Fold Resistance		x 0.1	1	1 0	100	10^3
AZT	=	0.5					
3TC	=	0.4					
Saquinavir	=	0.4					
Ritonavir	=	0.6					
Indinavir	=	0.8					

Fig. 10A

Resistance Level (Log units)

AZT
10,000
1,000
100
10
1

Indinavir

3TC

Ritonavir

Saquinavir

▢ Maximal Measurable Resistance

▨ Patient Sample Actual Resistance

▥ Reference Strain IIIB

Fig. 10B

17/18

Relative Change in Drug Susceptibility

 : Patient Resistance

 : Max. Measurable Resistance Increasing Resistance

 : standard dev. Normal range

DRUG	Fold Resistance	
		x 0.1 1 10 100 10^3
AZT	=	0.3
3TC	=	19
Saquinavir	=	1
Ritonavir	=	24
Indinavir	=	12

Fig. 11A

Resistance Level (Log units)

10,000 / 1,000 / 100 / 10 / 1

AZT, 3TC, Saquinavir, Ritonavir, Indinavir

 Maximal Measurable Resistance
 Patient Sample Actual Resistance
 Reference Strain IIIB

Fig. 11B

18/18

Fig. 12A

Fig. 12B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched - (classification system followed by classification symbols)
IPC 6   G01N   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 875 396 A (WEBB STEPHEN R) 1 April 1975 see figure 1	7
A,P	WO 96 08580 A (SEPRACOR INC ;MELNICK LAURENCE M (US); HEEFNER DONALD L (US)) 21 March 1996 see the whole document	1-29

☐ Further documents are listed in the continuation of box C.   ☒ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1997	24.06.97

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 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
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 Hoekstra, S |

Form PCT/ISA/210 (second sheet) (July 1992)

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WO 9608580 A	21-03-96	AU 3635295 A	29-03-96
		CA 2199805 A	21-03-96